Journal of Applied Bacteriology 1987, **62**, 71–80 2370/07/86

The growth and nutrition of *Clostridium sporogenes* NCIB 8053 in defined media

R.W. LOVITT, J.G. MORRIS & D.B. KELL *Department of Botany and Microbiology, University College of Wales, Aberystwyth, Dyfed SY23 3DA, Wales, UK*

Received 11 July 1986, revised 22 September 1986 and accepted 26 September 1986

LOVITT, R.W., MORRIS, J.G. & KELL, D.B. 1987. The growth and nutrition of *Clostridium sporogenes* NCIB 8053 in defined media. *Journal of Applied Bacteriology* **62**, 71–80.

Various defined and minimal media are described for the growth of *Clostridium sporogenes* NCIB 8053. The organism requires 10 amino acids and one vitamin for growth, whilst three other vitamins are growth stimulatory. L-α-hydroxy acid analogues can replace eight, and fatty acid analogues four, of these amino acids. The organism may generate free energy by a variety of Stickland reactions. Most Stickland acceptors, but not glycine, stimulate the growth of this organism on glucose. Nonetheless, cells grown in the presence of glycine will reductively deaminate it. The media described support the growth of several other strains of this species. The simplified growth media which we have developed permit quantitative studies of the physiology of this organism.

Proteolytic clostridia are a major causative agent of the spoilage of canned foods at neutral and slightly acidic pH, as this genus can form heat-resistant spores (Frazier & Westhoff 1978). *Clostridium sporogenes* in particular has been studied in this respect, not least because of its similarity to proteolytic, toxin-producing strains of *Cl. botulinum*. Nonetheless, most studies have concentrated upon the physical and chemical factors modulating the heat resistance, survival and germination of spores (e.g. Anema & Geers 1973; Frazier & Westhoff 1978).

It has been known since the 1930s that *Cl. sporogenes* is a proteolytic organism, in that it is capable of growth in media containing no carbohydrates (although these stimulate growth), and that, by inference, the organism is capable of obtaining the free energy required for growth by the utilization of amino acid mixtures alone. The fermentation of amino acids was first investigated by Stickland (1934), who discovered that certain pairs of amino acids may be transformed, and proposed that this type of reaction, which now bears his name (Nisman 1954; Barker 1961, 1981; Seto 1980), was the source of free energy for growth. Stickland (1935a,b) used washed cell suspensions to demonstrate that proline and glycine were reduced in the presence of other 'donor' amino acids or appropriate redox dyes, and that several other amino acids (alanine, valine, leucine and isoleucine) could be oxidized in the presence of oxidized dyes or the 'acceptor' amino acids proline or glycine.

Work by Quastel (cited by Mamelak & Quastel 1953) and by Nisman (1954) showed that the NAD/NADH couple played an important role as an intermediary electron carrier. The oxidation of amino acids is believed to occur by transmination to keto acids, which are then decarboxylated to form fatty acids, whilst free energy is thought to be conserved by substrate level phosphorylation *via* fatty acid kinase reactions (Nisman 1954; Barker 1961). The reducing equivalents so generated are used to reduce amino acids such as glycine, proline, ornithine and phenylalanine, reactions which, at least in the case of glycine (see Seto 1980), have also been shown to be coupled to phosphorylation. Bader *et al.* (1982) have confirmed and extended these findings in a recent study using *Cl. sporogenes*. Yet despite the above, few studies have been performed to assess the quantitative significance of these metabolic pathways to the growth of, and free energy conservation

in, *Cl. sporogenes* since most studies have been carried out in complex media, an approach which severely hinders the interpretation of physiological experiments.

The nutritional requirements of *Cl. sporogenes* additional to those involved in energy generation have been studied qualitatively by several workers (Fildes & Richardson 1935; Schull *et al.* 1949; Campbell & Frank 1956; Belokopytov *et al.* 1982; Monticello *et al.* 1984). However, quantitative data on these requirements are still lacking. Qualitatively, most strains examined require between eight and 11 amino acids (Campbell & Frank 1956; Belokopytov *et al.* 1982), in addition to those participating in the Stickland reaction. Several vitamin requirements, including biotin, folate, *p*-amino benzoate, thiamine and nicotinate have also been demonstrated (Schull & Peterson 1948; Campbell & Frank 1956; Belokopytov *et al.* 1982), as has a requirement for several metals (Costilow 1977). Carbon dioxide has also been shown either to be required or to stimulate the growth of this organism (Gladstone *et al.* 1935).

Before undertaking a quantitative study of the physiology of strain NCIB 8053 in chemostat culture, and motivated also by an awareness of the possible biotechnological importance of this genus in microbial biotransformations (Morris 1983), we sought to develop defined minimal media for the growth of the organism. The purpose of the present paper is therefore to report the nutritional requirements of *Cl. sporogenes* NCIB 8053 and the Stickland reactions which it undertakes. An accompanying paper (Lovitt *et al.* 1987) describes in more detail the metabolism and growth energetics of this organism when glucose is the carbon and electron source.

Materials and Methods

CHEMICALS

These were obtained from Sigma unless otherwise stated. Water was deionized and doubly distilled in an all-glass apparatus.

SOURCE AND MAINTENANCE OF ORGANISMS

Clostridium sporogenes strains 532, 8053 and 10697 were obtained from NCIB as freeze-dried cultures. Cultures were resuscitated on Reinforced Clostridial Medium (RCM, Lab M) in both broths and plates (containing 1·5% w/v agar). The organisms were freeze-dried in horse serum for long-term storage and were regularly subcultured on RCM, tryptone (0·5%)-yeast extract (0·3%)-glucose (0·5%) and EAA/FA-glucose medium (see below) at 37°C. Viable cultures could be maintained for over 2 months when stored at 4°C.

MEDIA

All media were based upon a low phosphate basal medium (LPBM) of the following composition (g/l): KH_2PO_4, 2·0; K_2HPO_4, 2·0; $MgCl_2.6H_2O$, 0·2; $(NH_4)_2SO_4$, 5·0; 10% $NaHCO_3$, 25 ml added after sterilization; 0·01% w/v resazurin solution, 1 ml; general vitamin solution, 10 ml added after sterilization; trace element solution, 10 ml. The trace element solution contained (g/l): nitrilotriacetic acid, 12·8; $FeSO_4.7H_2O$, 0·1; $MnCl_2.4H_2O$, 0·1; $CoCl_2.2H_2O$, 0·17; $CaCl_2.2H_2O$, 0·1; $ZnCl_2$, 0·1; $CuCl_2$, 0·01; H_3BO_4, 0·01; $NaMoO_4.2H_2O$, 0·01; NaCl, 1·0; $NaSeO_3$, 0·017; $NiSO_4.6H_2O$, 0·026; $NaWO_4.2H_2O$, 0·1. The general vitamin solution, which was sterilized by filtration, contained (mg/l): biotin, 2; folic acid, 2; pyridoxamine HCl, 10; thiamine HCl, 5; riboflavin HCl, 5; nicotinic acid, 5; pantothenic acid, 5; cyanocobalamin, 1; *p*-amino benzoic acid (PABA), 5; lipoic acid, 5. A simple vitamin solution, which was sterilized by autoclaving (121°C for 15 min), was also used, and contained the following (mg/l): thiamine HCl, 50; biotin, 5; PABA, 5; nicotinic acid, 500. The high nicotinic acid concentration maintained the pH at a low value and served to stabilize the thiamine to autoclaving. Where used, 1 ml of the simple vitamin solution was added to each litre of medium. The medium pH was adjusted to 7·0 before autoclaving; after the addition of the bicarbonate solution the pH was approx. 7·5. After all supplements were added, 5% w/v cysteine HCl (pH 6) was added until reduction was effected (as judged by the discolouration of the resazurin); this required *ca* 5–10 ml/l medium.

Standard amino acid complete (SACC) medium was LPBM with the addition of the following L-amino acids to a final concentration of 1 mmol/l each: glycine, valine, leucine, isoleucine, methionine, histidine, arginine, phenylalanine, tyrosine and tryptophan. Amino acid

complete (MACC) medium was LPBM supplemented with 3 mmol/l glycine, 2 mmol/l each of valine, isoleucine and arginine, 1 mmol/l each of leucine, histidine, methionine, phenylalanine and tryptophan and 0·125 mmol/l tyrosine. Essential amino acid/fatty acid (EAA/FA) medium was the same as MACC medium except that leucine, isoleucine and valine were replaced by 1 mmol/l each of 2-methyl propionic acid, 3-methyl butyric acid and 2-methyl butyric acid. Amino acids (except for 50 mmol/l tryptophan, which was filter-sterilized) were made up as a concentrated solution and sterilized by autoclaving. Neutralized fatty acids were made up as 40 mmol/l solutions and sterilized by autoclaving; yeast extract and tryptone were 10% w/v solutions. L-proline and glucose solutions (20% w/v) were sterilized separately and added to the appropriate concentrations.

CULTIVATION OF ORGANISMS

Organisms were grown in pressure tubes (Bellco, Vineland, NJ) containing 10 ml medium. The medium (LPBM) was dispensed anaerobically into the pressure tubes with a headspace of 'oxygen-free' (<4 ppm) nitrogen. The tubes were autoclaved and the other components added aseptically. Pre-reduced tubes were inoculated with 0·2 ml of culture with a syringe and incubated at 37°C without shaking. Larger volumes were cultured in 2 l Buchner flasks sealed with a rubber bung and fitted with a sample port, bladder and gas filters to allow flushing and evacuation of the flask headspace. The flasks, containing 1 or 1·5 l of medium, were autoclaved (121°C for 15 min) and after cooling, sterile supplements were added and the headspace was filled with N_2/CO_2 (95 : 5 v/v). Finally, the medium was pre-reduced with cysteine and inoculated with 5 ml of a 15 h pressure tube culture. Cultures were grown to mid-to-late log phase (0·4 g dry wt/l, *ca* 12–14 h after inoculation) and were harvested and washed with anaerobic buffer consisting of 50 mmol/l potassium phosphate buffer pH 7·0. The cells were resuspended in this buffer and stored anaerobically on ice.

ESTIMATION OF GROWTH

Growth was estimated by the measurement of optical density at 680 nm within the pressure tubes (1·8 cm path length). With this path length, 1 O.D. unit corresponded to 230 mg dry weight/l. For growth in flasks, samples were taken and read in cuvettes.

BIOTRANSFORMATIONS BY WASHED CELL SUSPENSIONS

Biotransformations were performed in 50 ml Wheaton bottles (Phase Separations, Queensferry) containing 10 ml of the anaerobic buffer referred to above plus 0·1% w/v cysteine HCl, cells (2–3 mg dry weight) and other components given in the legends to the tables, under a headspace of N_2 or N_2–H_2. Bottles were incubated in a gyrotary waterbath at 37°C. Gas and liquid samples were taken over a 2 h period with syringes, and liquid samples were acidified and stored frozen until analysed for glucose consumption and isobutyrate formation.

ESTIMATION OF FERMENTATION PRODUCTS

Gases and fatty acids were estimated using gas chromatography (Gottschal & Morris 1981), except that for the latter *n*-butanol or pentan-3-one were employed as internal standards. Glucose was estimated using a Sigma enzyme kit no. 510.

Results

AMINO ACID AND VITAMIN REQUIREMENTS

The first step in this study was to establish which (L-) amino acids were required for the growth of *Cl. sporogenes* NCIB 8053 for reasons independent of their role in the Stickland reaction. For this purpose we used a defined medium capable of supporting good growth, containing glucose (22 mmol/l) as a source of carbon and electrons plus proline (45 mmol/l) as electron acceptor, and to which were added a general vitamin solution (see Materials and Methods) and various amino acids. By a series of pool experiments it was established that only 10 amino acids were required. By using thioglycollate as a reducing agent it was established that cysteine was not required. Similarly proline, although stimulating growth considerably (and see later), was not an essential amino acid. As there is considerable variability in the amino

Table 3. The effect of dilution rate on the fermentation of glucose by *Clostridium sporogenes* in glucose-limited chemostat culture*

D (/h)	Biomass (g/l)	$q_{glucose}$ (mmol/(g h))	$Y_{x/s}$† (g/mol)	ATP_{glu} (mol/mol)	Fermentation products‡						Recovery of glucose carbon (%)
					Formate	Acetate	Ethanol	Butyrate	Butanol	CO_2	
0·095	0·262	2·43	39·1	3·7	0·18	10·1	0·42	1·94	0·56	15·3	114
					(3)	(150)	(6)	(29)	(8)		
0·133	0·268	3·32	40·0	3·6	0·31	10·2	0·13	0·77	0·29	12·5	93
					(5)	(152)	(2)	(12)	(4)		
0·163	0·307	3·55	45·8	3·8	0·31	11·0	0·31	1·06	0·44	14·0	102
					(5)	(160)	(4)	(15)	(6)		
0·200	0·323	4·15	48·2	3·6	0·36	9·8	0·27	0·90	0·35	12·2	94
					(5)	(146)	(4)	(13)	(5)		
0·225	0·329	4·58	49·1	3·8	0·37	10·7	0·45	1·32	0·55	14·5	111
					(5)	(159)	(7)	(20)	(8)		
0·246	0·339	4·86	50·6	3·8	0·50	11·1	0·36	0·86	0·33	13·3	103
					(7)	(166)	(5)	(12)	(5)		
0·270	0·342	5·29	51·0	3·6	0·81	9·8	0·51	0·72	0·26	11·5	92
				Mean 3·7	(12)	(146)	(7)	(11)	(4)		

* The experiments were performed in a chemostat with a total volume of 480 ml. The organisms were grown at pH 7·0, at 37°C in EAA medium containing 6·7 mmol/l glucose and the medium was gassed with CO_2. End products were determined as described in Materials and Methods, except that CO_2 was assessed from the other products and the known patterns of their production.

† $Y_{x/s}$, Specific yield of biomass, i.e. g dry wt of cells produced per mol glucose utilized.

‡ Values are given in mmol/l and, in parentheses, as mmol/l/100 mmol/l glucose utilized.

Table 2. Fatty acid replacements of amino acids required for the growth of *Clostridium sporogenes* NCIB 8053 in amino acid complete (MACC) medium

Amino acid omitted	Fatty acid analogue provided	Growth ($O.D._{680}^{18\,mm}$) No fatty acid	Plus fatty acid
Phenylalanine	Phenyl acetic	0·15	0·51
Tyrosine	Hydroxyphenylacetic	0·05	0·05
Tryptophan	Indole acetic	0·05	0·05
Valine	2-methyl propionic	0·02	1·50
Leucine	3-methyl butyric	0·02	2·00
Isoleucine	2-methyl butyric	0·03	1·50
Histidine	Imidazole 4 acetic	0·05	0·05
Arginine	Guanidobutyric	0·05	0·05
Glycine	Formic	0·05	0·05

Cultures were grown on MACC medium without the respective amino acid; for each condition organisms were grown in medium limited for the amino acid and then subcultured into the test medium and incubated anaerobically at 37°C for 24 h. Each subculture was made in duplicate and the results were averaged. Fatty acids were added at a concentration of 1 mmol/l.

growth experiments were performed in which each of the required amino acids in MACC medium was replaced by a 1 mmol/l supplement of the relevant fatty or L-α-hydroxy acid analogue. Cultures were inoculated from amino acid-limited batch cultures of the types used in Fig. 1. Table 1 shows that the relevant L-α-hydroxy acids could be used to replace all the amino acids required for growth except methionine (for which the appropriate analogue was unavailable) and glycine. Similar experiments were also performed with fatty acids, and the results are displayed in Table 2. Here valine, leucine, isoleucine and phenylalanine could be replaced with the relevant fatty acid analogues, although both growth yield and growth rate were significantly decreased when phenylacetate was used to replace phenylalanine (Tables 2 and 5). The effect of the concentration of the other three analogues on the growth of *Cl. sporogenes* is shown in Table 3, wherein it may be seen that 1 mmol/l concentrations gave maximum growth rates. It may be mentioned that the requirement for leucine, valine and isoleucine could not be satisfied by the relevant fatty acid analogues in which the carboxyl group was replaced by an amino or hydroxy group. Thus the defined medium designated essential amino acid/fatty acid (EAA/FA) was developed.

Table 3. Effect of fatty acid concentration in essential amino acid/fatty acid (EAA/FA) medium on the rate and extent of growth of *Clostridium sporogenes* NCIB 8053

Fatty acid concentration (mol/l (each))	Biomass ($O.D._{680}^{18\,mm}$)	Specific growth rate (per h)
0	0·07	0
50	0·37	0·12
100	0·70	0·13
150	0·98	0·20
200	1·20	0·29
400	1·40	0·42
1000	1·40	0·46
2000	1·40	0·48

The fatty acid solution contained three fatty acids: isobutyrate, isovalerate and 2-methyl butyrate in equal amounts. Organisms were grown in pressure tubes in EAA/FA medium with the fatty acids at the specified concentrations. A portion (0·2 ml) of a 16 h culture on EAA/FA medium was used as the inoculum.

A REQUIREMENT FOR ACETATE WHEN *Clostridium sporogenes* IS GROWN ON STICKLAND COUPLES

Attempts to grow the organisms on the Stickland couple valine/proline in EAA/FA were unsuccessful, due, it was assumed, to a requirement for carbon skeletons which this organism was unable to satisfy under these conditions. It was further assumed that such a requirement might be satisfied by the inclusion of acetate in the medium, which, *via* reductive carboxylation to pyruvate and thence *via* recognized pathways, could serve to generate the 3-, 4- and

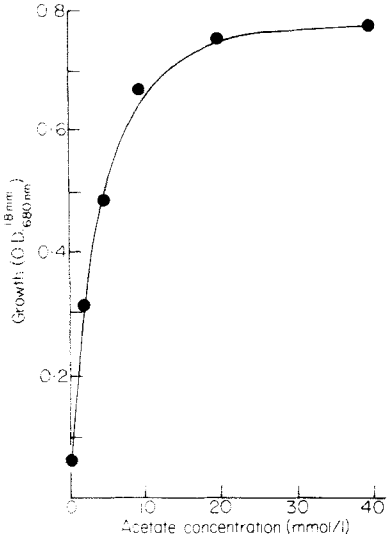

Fig. 2. The influence of acetate concentration on the growth of *Clostridium sporogenes* on valine-proline essential amino acid/fatty acid medium. Organisms were grown in pressure tubes and incubated anaerobically at 37°C for 48 h.

6-carbon skeletons normally derived from glucose. This was indeed found to be the case, and the organism was shown to have a requirement for acetate when the source of free energy for growth was a Stickland couple consisting of leucine, isoleucine or valine as electron donor and proline as electron acceptor; Fig. 2 demonstrates that growth was directly proportional to acetate concentration in EAA/FA medium for the valine/proline system.

ENERGY SOURCES AND THE EFFECTS OF PROLINE

The influence of L-proline on the fermentation of various carbon and energy sources is shown in Table 4. Whilst the organism utilized the sugars reported by Smith & Hobbs (1974) the efficiency of growth was somewhat variable. The organism was capable of good growth on sorbitol, sucrose and maltose, could ferment glycerol and lactate to a certain extent but could not use pyruvate. Growth was considerably increased in the presence of proline, however (and see Lovitt *et al.* 1987), especially with glycerol. Of the various amino acids tested, serine and, to a lesser extent, valine, leucine and isoleucine, were the only amino acids which could be fermented in the absence of proline. When proline was

Table 4. Effect of L-proline on the growth of *Clostridium sporogenes* NCIB 8053 on various carbon sources

Carbon source	Growth ($O.D._{680}^{18\,mm}$)		
	No C source	No proline	Plus proline
Pyruvate	0·06	0·06	0·06
Lactate	0·06	0·22	0·51
Glycerol	0·06	0·30	2·00
Glucose	0·05	0·07	1·50
Sorbitol	0·05	NT	0·56
Sucrose	0·05	NT	0·56
Maltose	0·05	NT	0·20
Serine	0·05	0·52	1·20
Valine	0·05	0·25	1·10
Leucine	0·04	0·15	1·10
Isoleucine	0·03	0·14	0·98
Norleucine	0·08	NT	0·50
Norvaline	0·06	NT	0·48
α-amino butyrate	0·05	0·05	0·05

The tubes contained essential amino acid/fatty acid (EAA/FA) medium which was inoculated with 0·25 ml of a culture from EAA/FA-glucose medium and then incubated anaerobically at 37°C for 48 h. Carbon sources were added to a final concentration of 20 mmol/l and L-proline to a concentration of 45 mmol/l.

NT, Not tested.

present, norleucine (2-amino hexanoate) and norvaline (2-amino valerate) could also serve as energy sources. Thus the substrate range of this organism can be significantly enhanced by the addition of proline (and other electron acceptors), a point which is of some taxonomic and ecological significance. D-proline could also serve as electron acceptor, and is actually the substrate of proline reductase; however, these organisms contain an active proline racemase which, together with the D-proline reductase, serves to reduce either D- or L-proline to 5-amino pentanoic acid. Growth could not be supported by glutamate, aspartate, histidine, threonine, arginine, lysine, tryptophan, phenylalanine or methionine, whether proline was present or not.

Table 5 shows the growth rate and growth yield of this organism in a variety of minimal, defined and complex media. It may first be mentioned that a certain amount of adaptation to these various energy sources is required, in that at least two subcultures were required to obtain cultures which manifested the growth rates and growth yields given. It is of particular interest that glycine appears to exert only a marginal stimulation of growth when compared with

Table 5. Extent and rate of growth of *Clostridium sporogenes* NCIB 8053 on defined and complex media

Medium	Final $O.D._{680}^{18\,mm}$	Final pH	Doubling time (h)
Complex			
TYE	1·10	7·15	1·04
TYE/glucose	1·50	6·14	0·83
TYE/Pro	1·50	6·90	0·99
Defined			
MACC	0·35	7·24	4·22
MACC/glucose	0·70	6·54	2·55
MACC/glucose/Pro	1·50	5·29	1·28
EAA/FA/glucose/Pro − Phe + phenylacetate	0·80	5·41	4·25
EAA/FA/glucose/Pro	1·50	5·35	1·35
EAA/FA/glucose/Orn	1·10	6·12	1·75
EAA/FA/glucose/Phe	1·70	5·75	1·60
EAA/FA/glucose/Gly	0·64	6·35	2·10
EAA/FA/glucose	0·66	6·35	2·01
EAA/FA/valine/Pro	1·10	6·82	1·78
EAA/FA/valine/Orn	0·68	6·72	2·92
EAA/FA/valine/Phe	0·68	6·84	3·10
EAA/FA/valine/Gly	0·40	6·88	3·40
EAA/FA/valine	0·25	6·91	3·11
EAA/FA/leucine/Pro	1·10	ND	ND
EAA/FA/leucine/Gly	0·32	ND	ND
EAA/FA/leucine	0·27	ND	ND
EAA/FA/isoleucine/Pro	0·98	ND	ND
EAA/FA/isoleucine/Gly	0·30	ND	ND
EAA/FA/isoleucine	0·27	ND	ND

Organisms were grown at 37°C in unshaken sealed tubes containing 10 ml of culture medium. Growth curves were obtained by observing the change in O.D. with time, measurements being taken directly from the tubes (18 mm light path) at 680 nm. Values given are the means of triplicates.

The electron donors glucose (27 mmol/l), valine (20 mmol/l), leucine (20 mmol/l) and isoleucine (20 mmol/l) were present at the concentrations indicated, whilst proline (Pro) and other electron acceptors (ornithine (Orn), phenylalanine (Phe), glycine (Gly)) additions were to 45 mmol/l final concentrations.

TYE, LPBM + 1% tryptone and 0·3% yeast extract; MACC medium, amino acid complete; EAA/FA, essential amino acids (as MACC medium but valine was replaced by 1 mmol/l isobutyrate, leucine by 1 mmol/l isovalerate and isoleucine by 1 mmol/l 2-methyl butyrate). For further details see text.

ND, Not determined.

proline, phenylalanine or ornithine as the electron acceptor in a Stickland or 'mixed Stickland' (carbohydrate as electron donor) couple. Despite the poor growth, however, analysis of the culture media indicated that the addition of glycine did stimulate the formation of isobutyrate (from valine), 3-methyl butyrate (from leucine) and 2-methyl butyrate (from isoleucine), and this phenomenon was studied further as outlined below. The following compounds did not serve as electron acceptors: betaine, arginine, nitrate, thiosulphate, sulphite, sulphate or fumarate.

A COMPARISON OF *Clostridium sporogenes* STRAINS ON MACC MEDIUM

It was observed that all strains of *Cl. sporogenes* tested were able to grow on the MACC medium, although qualitative differences in the pattern of sugar utilization were observed as shown in Table 7. The major end products of glucose dissimilation when proline was present were acetate and ethanol (Table 7). It may be mentioned that, as discussed also by Princewill (1978), the different colony morphologies of these strains on rich medium could be

Table 6. Comparison of the growth of three strains of *Clostridium sporogenes* on amino acid complete (MACC) medium

	Growth $O.D._{680}^{18\,mm}$		
	NCIB strain		
	8053	532	10697
Requirement for complete MACC medium	+	+	+
Carbohydrate utilized			
Glucose	0·95	0·78	0·74
Sorbitol	0·56	0·42	0·20
Salicin	0·56	0·08	0·05
Sucrose	0·35	0·17	0·12
Lactate	0·75	0·16	0·13
Maltose	0·20	0·48	0·48
Glycerol	0·70	0·88	0·72
End products (mmol/l) from glucose (27 mmol/l) after 24 h			
Acetate	20	22	25
Ethanol	25	26	23
Butyrate	Trace	Trace	Trace
Butanol	–	–	–

Organisms were grown as described in the text, where further details are given.

Table 7. Stickland reactions undertaken by washed suspensions of *Clostridium sporogenes* NCIB 8053 grown under various conditions

	Rate of substrate consumption or product formation (nmol/min/mg dry wt)					
	Glucose/proline grown cells		Valine/proline grown cells		Complex medium grown cells	
Valine consumption						
Valine	2		9		4	
Valine + glycine	4		33		23	
Valine + proline	115		118		35	
Glucose consumption						
Glucose	18		ND		ND	
Glucose + glycine	16		ND		ND	
Glucose + proline	78		ND		ND	
Hydrogen consumption						
Hydrogen + glycine	ND		ND		ND	
Hydrogen + proline	ND		ND		ND	
	H_2	(CO_2)	H_2	(CO_2)	H_2	(CO_2)
Hydrogen and carbon dioxide production						
Valine	0·38	ND	2·78	ND	0·54	+
Valine + glycine	ND	+	ND	+	ND	+
Valine + proline	0·44	+	3·18	+	1·68	+
Glucose	0·38	+	ND	ND	ND	+
Glucose + glycine	0·66	+	ND	ND	ND	+
Glucose + proline	0·38	+	ND	ND	ND	+

Organisms were grown in the media described to mid/late exponential phase of culture growth. They were then harvested, washed twice with phosphate buffer pH 7·0 and transferred to a 50 ml bottle containing 10 ml reduced phosphate buffer plus substrates under a nitrogen gas phase. The suspension was incubated for 2 h at 37°C. Samples were taken periodically and the rates of substrate consumption and product formation were determined. Added substrates were glucose (10 mmol/l), valine (37 mmol/l), glycine (133 mmol/l) and proline (86 mmol/l).

ND, Not detectable.

correlated with their ability to ultilize maltose in that whilst strains 532 and 10697 formed rough rhizoid colonies on RCM, strain 8053 gave smooth colonies with slightly irregular margins.

THE STICKLAND REACTION IN WASHED CELL SUSPENSIONS OF *Clostridium sporogenes* NCIB 8053 GROWN ON VARIOUS DEFINED MEDIA

Observations made by others (Stickland 1935a; Woods 1936; Mamelak & Quastel 1953; Venugopalan 1980) have noted that cell suspensions of *Cl. sporogenes* are capable of reducing glycine (to acetate and ammonia). However, we found that little or no growth stimulation was obtained when glycine was used as electron acceptor. Thus an experiment was designed to determine the influence of the growth substrate on the rates of glucose and amino acid transformations by washed cells, with a view both to confirming the earlier observations and to checking the ability or otherwise of our strain to transform glycine. The results of this study are given in Table 7. It may be seen that glucose dissimilation was not observed in cells which had not been grown on this substrate and that glucose or valine grown cells had a much decreased ability to reduce glycine as compared with proline. The ability of these different types of cells to produce hydrogen followed a similar pattern. In contrast to some strains (Hoogerheide & Kocholaty 1938; Bader & Simon 1983), our strain did not consume hydrogen when presented with an electron acceptor (Table 7). Thus a significant rate limitation to balanced growth appeared to be associated with the re-oxidation of reducing equivalents produced by the oxidation of glucose or donor amino acids.

Discussion

We have developed a defined medium for the growth of *Cl. sporogenes* NCIB 8053, with a view to defining in more quantitative terms than heretofore its requirements for carbon and energy sources and for vitamins and amino acid supplements. We found that 10 amino acids were essential for growth, a number typical of those previously published (Campbell & Frank 1967; Gottschalk *et al.* 1981; Belokopytov *et al.* 1982). The problem of amino acid synthesis in this organism is apparently that of generating appropriate carbon skeletons, presumably *via* keto acids (Golovchenko *et al.* 1983). Branched chain amino acids may be replaced by the fatty acid analogues, which may be transformed to the appropriate keto acid by reductive carboxylation (Buchanan 1972; Monticello & Costilow 1982; Monticello *et al.* 1984), although the interchangeability of amino acids noted by these workers was not observed. An interesting observation was the ability of hydroxy acids to fulfill most of the requirements of this organism for amino acids. Together, these observations imply that the organisms have a general L-α-hydroxy acid dehydrogenase and transaminase(s), as has been suggested by the work of Bader *et al.* (1982), O'Neil & DeMoss (1968) and of Jean & DeMoss (1968), respectively.

By the use of the present defined medium, we were able to demonstrate clearly the extent to which this organism can use the Stickland reaction to derive the free energy required for growth. It was of particular interest that glycine was a very poor substitute for proline as an electron acceptor for growth. This is not due, for instance, to a lack of selenium (Turner & Stadtman 1973; Costilow 1977), since adequate amounts were present in the medium and doubling its concentration was without effect (results not shown). A comparison of the growth rates of this organism on various substrates indicated the importance of maintaining an appropriate redox balance. Studies with washed cell suspensions showed that their ability to reduce exogenous glycine was significantly less than their capacity to reduce proline, a phenomenon which would simply account for the inability of this strain to grown on glycine as a Stickland acceptor. More quantitative studies unavoidably require the assessment of fermentation end products and growth in chemostat culture. Such studies are described in an accompanying article.

We thank the Biotechnology Directorate of the Science and Engineering Research Council, UK, for financial support, and Jane Watts for typing the manuscript.

References

ANEMA, P.J. & GEERS, J.M. 1973 Controlling the sporulation of *Clostridium sporogenes* and the heat resistance of spores. *Journal of Applied Bacteriology* **36**, 553–558.

BADER, J. & SIMON, H. 1983 ATP formation is coupled to the hydrogenation of 2-enoates in *Clostridium sporogenes*. *FEMS Microbiology Letters* **20**, 171–175.

BADER, J., RAUSCHENBACH, P. & SIMON, H. 1982 On a hitherto unknown fermentation path of several amino acids by proteolytic clostridia. *FEBS Letters* **140**, 67–72.

BARKER, H.A. 1961 Fermentations of nitrogenous organic compounds. In *The Bacteria, Vol II Metabolism* ed. Gunsalus, I.C. & Stanier, R.Y. pp. 151–208. London: Academic Press.

BARKER, H.A. 1981 Amino acid degradation by anaerobic bacteria. *Annual Review of Biochemistry* **50**, 23–40.

BELOKOPYTOV, B.F., GOLOVCHENKO, N.P., KRAUZOVA, V.I., CHUVILSKAYA, N.A., AKIMENIO, V.K. & LOZINOV, A.B. 1982 A chemically defined medium for cultivation of *Clostridium sporogenes*. *Mikrobiologiya* **51**, 354–361.

BUCHANAN, B.B. 1972 Ferredoxin-linked carboxylation reactions. In *The Enzymes*, Vol. 4, 3rd edn. ed. Boyer, P.D. pp. 193–216. New York: Academic Press.

CAMPBELL, L.L. & FRANK, H.A. 1956 Nutritional requirements of some putrefactive anaerobic bacteria. *Journal of Bacteriology* **71**, 267–269.

COSTILOW, R.N. 1977 Selenium requirement for the growth of *Clostridium sporogenes* with glycine as the oxidant in the Stickland reaction systems. *Journal of Bacteriology* **131**, 366–368.

FILDES, P. & RICHARDSON, G.M. 1935 The amino acids necessary for the growth of *Cl. sporogenes*. *British Journal of Experimental Pathology* **16**, 326–335.

FRAZIER, W.C. & WESTHOFF, D.C. 1978 *Food Microbiology*, 3rd edn. New York: McGraw-Hill.

GLADSTONE, G.P., FILDES, P. & RICHARDSON, G.M. 1935 Carbon dioxide as an essential factor in the growth of bacteria. *British Journal of Experimental Pathology* **16**, 335–348.

GOLOVCHENKO, N.P., BELOKOPYTOV, B.F. & AKIMENKO, V.F. 1983 Transdeamination in bacteria *Clostridium sporogenes* and *Cl. sticklandii*. *Microbiology* **52**, 181–186.

GOTTSCHAL, J.C. & MORRIS, J.G. 1981 Nonproduction of acetone and butanol by *Clostridium acetobutylicum* during glucose- and ammonium-limitation in continuous culture. *Biotechnology Letters* **3**, 525–530.

GOTTSCHALK, G., ANDREESEN, J.R. & HIPPE, H. 1981 The genus *Clostridium* (non medical aspects). In *The Prokaryotes*, Vol. 2. ed. Starr, M.P., Stolp, M.P., Truper, H.G., Barlows, A. & Schlegel, H.G. pp. 1767–1803. Berlin: Springer-Verlag.

HOOGERHEIDE, J.C. & KOCHOLATY, W. 1938 Metabolism of the strict anaerobes (genus *Clostridium*) II. Reduction of amino acids with gaseous hydrogen by suspensions of *Cl. sporogenes*. *Biochemical Journal* **32**, 949–957.

JEAN, M. & DEMOSS, R.D. 1968 Indolelactate dehydrogenase from *Clostridium sporogenes*. *Canadian Journal of Microbiology* **14**, 429–435.

LOVITT, R.W., KELL, D.B. & MORRIS, J.G. 1987 The physiology of *Clostridium sporogenes* NCIB 8053 growing in defined media. *Journal of Applied Bacteriology* **62**, 81–92.

MAMELAK, R. & QUASTEL, J.H. 1953 Amino acid interactions in strict anaerobes (*Cl. sporogenes*). *Biochimica et Biophysica Acta* **12**, 103–120.

MONTICELLO, D.J. & COSTILOW, R.N. 1982 Interconversion of valine and leucine by *Clostridium sporogenes*. *Journal of Bacteriology* **152**, 946–949.

MONTICELLO, D.J., HADIOETOMO, R.S. & COSTILOW, R.N. 1984 Isoleucine synthesis by *Clostridium sporogenes* from propionate or α-methyl-butyrate. *Journal of General Microbiology* **130**, 309–318.

MORRIS, J.G. 1983 Anaerobic fermentations—some new possibilities. In *Biotechnology* ed. Phelps, C.F. & Clarke, P.H. pp. 147–172. London: The Biochemical Society.

NISMAN, B. 1954 The Stickland reaction. *Bacteriological Reviews* **18**, 16–42.

O'NEIL, S.R. & DEMOSS, R.D. 1968 Tryptophan transaminase from *Clostridium sporogenes*. *Archives of Biochemistry & Biophysics* **127**, 361–369.

PRINCEWILL, T.J.T. 1978 Differences in colony morphology and carbohydrate fermentation of *Clostridium sporogenes*. *Journal of General Microbiology* **108**, 315–319.

SCHULL, G.M. & PETERSON, W.H. 1948 The nature of the 'sporogenes vitamin' and other factors in the nutrition of *Clostridium sporogenes*. *Archives of Biochemistry* **18**, 69–83.

SCHULL, G.M., THOMA, R.W. & PETERSON, W.H. 1949 Amino acid and unsaturated fatty acid requirements of *Clostridium sporogenes*. *Archives of Biochemistry* **20**, 227–241.

SETO, B. 1980 The Stickland reaction. In *Diversity of Bacterial Respiratory Systems*, Vol. 2. ed. Knowles, C.J. pp. 50–64. Boca Raton, Florida: CRC Press.

SMITH, L.D.S. & HOBBS, G. 1974 Genus III *Clostridium*. In *Bergey's Manual of Determinative Bacteriology*, 7th edn. pp. 551–572. Baltimore: Williams & Wilkins.

STICKLAND, L.H. 1934 Studies in the metabolism of the strict anaerobes (genus *Clostridium*) I. The chemical reactions by which *Cl. sporogenes* obtains its energy. *Biochemical Journal* **28**, 1746–1759.

STICKLAND, L.H. 1935a Studies in the metabolism of the strict anaerobes (genus *Clostridium*). III. The oxidation of alanine by *Cl. sporogenes*. IV. The reduction of glycine by *Cl. sporogenes*. *Biochemical Journal* **29**, 889–898.

STICKLAND, L.H. 1935b Studies in the metabolism of strict anaerobes (genus *Clostridium*) II. The reduction of proline by *Cl. sporogenes*. *Biochemical Journal* **29**, 288–290.

TURNER, D.C. & STADTMAN, T.C. 1973 Purification of protein components of the clostridial glycine reductase system and characterisation of protein A as a selenoprotein. *Archives of Biochemistry and Biophysics* **154**, 366–381.

VENUGOPALAN, V. 1980 Influence of growth conditions on glycine reductase of *Clostridium sporogenes*. *Journal of Bacteriology* **141**, 386–388.

WOODS, D.D. 1936 Studies in the metabolism of strict anaerobes (genus *Clostridium*). V. Further experiments on the coupled reactions between pairs of amino acids induced by *Cl. sporogenes*. *Biochemical Journal* **30**, 1934–1936.